EXHIBIT B14

Reproductive Sciences

Reproductive Sciences

Molecular basis supporting the association of talcum powder use with increased risk of ovarian cancer

Journal:	*Reproductive Sciences*
Manuscript ID	RSCI-18-671.R1
Manuscript Type:	Original Manuscripts
Date Submitted by the Author:	03-Jan-2019
Complete List of Authors:	Fletcher, Nicole; Wayne State University School of Medicine, Obstetrics and Gynecology Harper, Amy; Wayne State University School of Medicine, Obstetrics and Gynecology Memaj, Ira; Wayne State University School of Medicine, Obstetrics and Gynecology Fan, Rong; Wayne State University School of Medicine, Obstetrics and Gynecology Morris, Robert ; Wayne State University School of Medicine, Obstetrics and Gynecology Saed, Ghassan; Wayne State University School of Medicine, Obstetrics and Gynecology
File Designation:	
Keyword:	talcum powder, oxidative stress, single nucleotide polymorphism, cell proliferation, epithelial ovarian cancer

Molecular basis supporting the association of talcum powder use with increased risk of ovarian cancer.

Nicole M. Fletcher, Ph.D.[a], Amy K. Harper, M.D.[ab], Ira Memaj, B.S.[a], Rong Fan, M.S.[a], Robert T. Morris, M.D.[b], Ghassan M. Saed, Ph.D.[a]

[a]Department of Obstetrics and Gynecology, Wayne State University School of Medicine and [b]Department of Gynecologic Oncology, Karmanos Cancer Institute, Detroit, MI

Corresponding Author:

Ghassan M. Saed, Ph.D.

Associate Professor of Gynecologic Oncology

Director of Ovarian Cancer Biology Research

Departments of Obstetrics and Gynecology and Oncology

Member of Tumor Biology and Microenvironment Program

Karmanos Cancer Institute

Wayne State University School of Medicine

Detroit, MI 48201

(313) 577-5433 Office phone

(313) 577-8544 Office fax

(313) 577-1302 Lab phone

gsaed@med.wayne.edu

Abstract

Genital use of talcum powder and its associated risk of ovarian cancer is an important controversial topic. Epithelial ovarian cancer (EOC) cells are known to manifest a persistent pro-oxidant state. Here we demonstrated that talc induces significant changes in key redox enzymes and enhances the pro-oxidant state in normal and EOC cells. Using real-time RT-PCR and ELISA, levels of CA-125, caspase-3, nitrate/nitrite, and selected key redox enzymes, including myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR) were determined. TaqMan® Genotype analysis utilizing the QuantStudio 12K Flex was used to assess single nucleotide polymorphisms in genes corresponding to target enzymes. Cell proliferation was determined by MTT Proliferation Assay. In all talc treated cells, there was a significant dose-dependent increase in pro-oxidants iNOS, nitrate/nitrite, and MPO with a concomitant decrease in anti-oxidants CAT, SOD, GSR, and GPX ($p<0.05$). Remarkably, talc exposure induced specific point mutations that are known to alter the activity in some of these key enzymes. Talc exposure also resulted in a significant increase in inflammation as determined by increased tumor marker CA-125 ($p<0.05$). More importantly, talc exposure significantly induced cell proliferation and decreased apoptosis in cancer cells and to greater degree in normal cells ($p<0.05$). These findings are the first to confirm the cellular effect of talc and provide a molecular mechanism to previous reports linking genital use to increased ovarian cancer risk.

Keywords: talc, epithelial ovarian cancer, oxidative stress, single nucleotide polymorphism, cell proliferation

Introduction

Ovarian cancer is the most lethal gynecologic malignancy and ranks fifth in cancer deaths among women diagnosed with cancer[1]. Epithelial ovarian cancer (EOC) has long been considered a heterogeneous disease with respect to histopathology, molecular biology, and clinical outcome[1, 2].

Although surgical techniques and treatments have advanced over the years, the prognosis of EOC remains poor, with a 5-year survival rate of 50% in advanced stage[2]. This is largely due to the lack of early warning symptoms and screening methods, and the development of chemoresistance[1, 2]. Moreover, ovarian cancer is known to be associated with germline mutations in the BRCA1 or BRCA2 genes, but with a rate of only 20-40%, suggesting the presence of other unknown mutations in other predisposition genes[3]. Additional genetic variations including single nucleotide polymorphisms (SNPs) have been hypothesized to act as low to moderate penetrant alleles that contribute to ovarian cancer risk[3, 4].

The pathophysiology of EOC is not fully understood but has been strongly associated with inflammation and the resultant oxidative stress[5]. We have previously characterized EOC cells to manifest a persistent pro-oxidant state as evident by the upregulation of key oxidants and downregulation of key antioxidants, which is further enhanced in chemoresistant EOC cells[6]. The expression of key pro-oxidant/inflammatory enzymes such as inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, and myeloperoxidase (MPO), as well as an increase in nitric oxide (NO) levels were increased in EOC tissues and cells[7]. Additionally, we have shown that EOC cells manifest lower apoptosis, which was markedly induced by inhibiting iNOS, indicating a strong link between apoptosis and NO/iNOS pathways in these cells[7].

The cellular redox balance is maintained by key antioxidants including catalase (CAT), superoxide dismutase (SOD) or by glutathione peroxidase (GPX) coupled with glutathione reductase (GSR)[5]. Other important scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which utilizes glutathione (GSH) as a substrate[7]. We have previously reported that a genotype switch in key antioxidants is a potential mechanism leading to the acquisition of chemoresistance in EOC cells[7]. We have studied the effects of genetic polymorphisms in key redox genes on the acquisition of the oncogenic phenotype in EOC cells, including genes that control the levels of cellular ROS and oxidative damage and SNPs for genes involved in carcinogen metabolism (detoxification and/or activation), antioxidants, and DNA repair pathways[4, 7]. Several function-altering SNPs have been identified in key antioxidants, including CAT, GPX, GSR, and SOD[4].

Several studies have suggested the possible association between genital use of talcum powder and risk of EOC[7-9]. Association between the use of cosmetic talc in genital hygiene and ovarian cancer was first described in 1982 by Cramer, et al, and many subsequent studies supported this finding[7]. Talc and asbestos are both silicate minerals; the carcinogenic effects of asbestos have been extensively studied and documented in the medical literature[7, 8]. Asbestos fibers in the lung initiate an inflammatory and scarring process, and it has been proposed that ground talc, as a foreign body, might initiate a similar inflammatory response[7]. The objective of this study was to determine the effects of talcum powder on the expression of key redox enzymes, CA-125 levels, and cell proliferation and apoptosis in normal and EOC cells.

Material and Methods

Cell Lines. Ovarian cancer cells SKOV-3 (ATCC), A2780 (Sigma Aldrich, St. Louis, MO), and TOV112D (a kind gift from Gen Sheng Wu at Wayne State University, Detroit, MI) and normal cells human macrophages (EL-1, ATCC, Manassas, VA), human primary normal ovarian epithelial cells (Cell Biologics, Chicago, IL), human ovarian epithelial cells (HOSEpiC, ScienCell Research Laboratories, Inc., Carlsbad, CA), immortalized human fallopian tube secretory epithelial cells (FT33, Applied Biological Materials, Richmond, BC, Canada) were used. All cells were grown in media and conditions following manufacturer's protocol. EL-1 cells were grown in IMDM media (ATCC) supplemented with 0.1 mM hypoxanthine and 0.1 mM thymidine solution (H-T, ATCC) and 0.05mM β-mercaptoethanol. SKOV-3 EOC cells were grown in HyClone McCoy's 5A medium (Fisher Scientific, Waltham, MA), A2780 EOC cells were grown in HyClone RPMI-1640 (Fisher Scientific), and both TOV112D EOC cells were grown in MCDB105 (Cell Applications, San Diego, CA) and Medium 199 (Fisher Scientific) (1:1). All media was supplemented with fetal bovine serum (FBS, Innovative Research, Novi, MI) and penicillin/streptomycin (Fisher Scientific), per their manufacturer specifications. Human primary normal ovarian epithelial cells were grown in Complete Human Epithelial Cell Medium (Cell Biologics).

Treatment of cells. Talcum powder (Fisher Scientific, Catalog #T4-500, Lot#166820) or baby powder (Johnson & Johnson, #30027477, Lot#13717RA) was dissolved in DMSO (Sigma Aldrich) at a concentration of 500 mg in 10 ml and was filtered with a 0.2 µm syringe filter (Corning). Sterile DMSO was used as a control for all treatments. Cells were seeded in 100 mm cell culture dishes (3×10^6) and were treated 24 hours later with 5, 20, or 100 µg/ml of talc for 72 hours. Cell pellets were collected for RNA, DNA, and protein extraction. Cell culture media was collected for CA-125 analysis by ELISA.

Real-Time RT-PCR. Total RNA was extracted from all cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). Measurement of the amount of RNA in each sample was performed using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). A 20 µL cDNA reaction volume containing 0.5 µg RNA was prepared using the SuperScript VILO Master Mix Kit (Life Technologies, Carlsbad, CA). Optimal oligonucleotide primer pairs were selected for each target using Beacon Designer (Premier Biosoft, Inc., Table 1). Quantitative RT-PCR was performed using the EXPRESS SYBR GreenER qPCR Supermix Kit (Life Technologies) and the Cepheid 1.2f Detection System as previously described[6]. Standards with known concentrations and lengths were designed specifically for *β-actin* (79 bp), *CAT* (105 bp), *NOS2* (89 bp), *GSR* (103 bp), *GPX1* (100 bp), *MPO* (79 bp), and *SOD3* (84 bp), allowing for construction of a standard curve using a 10-fold dilution series[6]. All samples were normalized to *β-actin*. A final melting curve analysis was performed to demonstrate specificity of the PCR product.

Protein Detection. Cell pellets were lysed utilizing cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin) containing a cocktail of protease inhibitors. Samples were centrifuged at 13000 rpm for 10 minutes at 4°C. Total protein concentration of cell lysates from control and talc treated cells was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Detection of Protein/Activity by ELISA. The following ELISA kits were used (Cayman Chemical, Ann Arbor, MI): CAT, SOD, GSR, GPX, and MPO. Nitrite (NO_2^-)/nitrate (NO_3^-) were determined

spectrophotometrically by measuring their absorbance at 210 nm after separation by HPLC with standard NO_2^-/NO_3^- as previously reported[6]. The analysis was performed by a HPLC system (Shimadzu Scientific Instruments, Inc.) including a LC-10ADV pump, frc-10A injector and DGU-14A degasser. Nitrite/nitrate was detected using an RF-10 XL fluorescence detector with 210 nm excitation and 440 nm emission. CA-125 protein levels were measured in cell media by ELISA (Ray Biotech, Norcross, GA).

TaqMan® SNP Genotyping Assay. DNA was isolated utilizing the EZ1 DNA Tissue Kit (Qiagen) for EOC cells. The TaqMan® SNP Genotyping Assay Set (Applied Biosystems, Carlsbad, CA) (NCBI dbSNP genome build 37, MAF source 1000 genomes) were used to genotype the SNPs (Table 1). The Applied Genomics Technology Center (AGTC, Wayne State University, Detroit, MI) performed these assays. Analysis was done utilizing the QuantStudioTM 12 K Flex Real-Time PCR System (Applied Biosystems).

Cell Proliferation and Apoptosis. Cell proliferation was assessed with the TACS MTT Cell Proliferation Assay (Trevigen, Gaithersburg, MD) after treatment with talc (100 µg/mL) for 72 hours. The Caspase-3 Colorimetric Activity Assay Kit (Chemicon, Temecula, CA) was used to determine levels of caspase-3 activity after treatment of normal and EOC cells with various doses of talc as previously described[7]. Equal concentrations of cell lysate were used. The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. The free pNA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with its control allows determination of the percent increase in caspase-3 activity.

Statistical Analysis. Normality was examined using the Kolmogorov-Smirnov test and by visual inspection of quantile-quantile plots. Because most of the data were not normally distributed, differences in distributions were examined using the Kruskal-Wallis test. Generalized linear models were fit to examine pairwise differences in estimated least squares mean expression values by exposure to 0, 5, 20 or 100 µg/ml of talc. We used the Tukey-Kramer adjustment for multiple comparisons, and the regression models were fit using log2 transformed analyte expression values after adding a numeric constant

'1' to meet model assumptions while avoiding negative transformed values. P-values below 0.05 are statistically significant.

Results

Talc treatment decreased the expression of antioxidant enzymes SOD and CAT in normal and EOC cells. Real-time RT-PCR and ELISA assays were utilized to determine the CAT and SOD mRNA and protein levels in cells before and after 72 hours talc treatment, respectively (Figure 1). CAT (Figure 1. A, C) and SOD (Figure 1. B, D) mRNA and protein levels were significantly decreased in a dose-dependent manner in talc treated cells compared to controls, ($p<0.05$).

Talc treatment increased the expression of prooxidants iNOS, NO_2^-/NO_3^- and MPO in normal and EOC cells. Real-time RT-PCR and NO_2^-/NO_3^- assays were utilized to determine the iNOS mRNA and NO levels in cells before and after 72 hours talc treatment, respectively (Figure 2). iNOS mRNA and NO levels were significantly increased in a dose-dependent manner in talc treated cells as compared to their controls (Figure 2. A, C, $p<0.05$). As expected, there was no detectable MPO in normal ovarian and fallopian tube cells, and thus talc treatment did not have any effect. However, MPO mRNA and protein levels were significantly increased in a dose-dependent manner in talc treated ovarian cancer cells and macrophages compared to controls (Figure 2. B, D, $p<0.05$).

Talc treatment decreased the expression of antioxidant enzymes, GPX and GSR in normal and EOC cells. Real-time RT-PCR and ELISA assays were utilized to determine the GPX and GSR mRNA and protein levels in cells before and after 72 hours talc treatment, respectively (Figure 3). GPX (Figure 3. A, C,) and GSR (Figure 3. B, D) mRNA and protein levels were significantly decreased in a dose-dependent manner in talc treated cells compared to controls ($p<0.05$).

Talc exposure induced known genotype switches in key oxidant and antioxidant enzymes. Talc treatment was associated with a genotype switch in *NOS2* from the common C/C genotype in untreated cells to T/T, the SNP genotype, in talc treated cells, except in A2780 and TOV112D (Table 2). Additionally, the observed decrease in CAT expression and activity was associated with a genotype

switch from common C/C genotype in CAT in untreated cells to C/T, the SNP genotype, in TOV112D and all normal talc treated cells. However, there was no detectable genotype switch in CAT in A2780, SKOV3, and TOV112D (Table 2). Remarkably, there was no observed genotype switch in the selected SNP for SOD3 and GSR in all talc treated cells. All cells except for HOSEpiC cells manifest the SNP genotype of *GPX1* (C/T). Intriguingly, talc treatment reversed this SNP genotype to the normal genotype (Table 2).

Talc treatment increased CA-125 levels in normal and EOC cells. CA-125 ELISA assay was performed in protein isolated from cell media before and after talc treatment. CA-125 levels were significantly increased in a dose-dependent manner in all cells (Figure 4, $p<0.05$). There was no detectable CA-125 protein in macrophages.

Talc treatment increased cell proliferation and decreased apoptosis. MTT Cell Proliferation Assay was used to determine cell viability, and Caspase-3 Activity assay was utilized to determine apoptosis of all cell lines after 72 hours talc treatment (Figure 5). Cell proliferation was significantly increased from the baseline in all talc treated cells ($p<0.05$), but to a greater degree in normal as compared to cancer cells. As anticipated, caspase-3 was significantly reduced in cancer as compared to normal cells. Talc treatment resulted in decreased caspase-3 activity in all cells as compared to controls (Figure 6, $p<0.05$), indicating a decrease in apoptosis.

Discussion

The claim that regular use of talcum powder for hygiene purpose is associated with an increased risk of ovarian cancer is based on several reports confirming the presence of talc particles in the ovaries and other parts of the female reproductive tract as well as in lymphatic vessels and tissues of the pelvis[7, 8]. The ability of talc particles to migrate through the genital tract to the distal fallopian tube and ovaries is well accepted[7, 8]. To date, the exact mechanism is not fully understood, though several studies have pointed toward the peristaltic pump feature of the uterus and fallopian tubes, which is known to enhance transport of sperm into the oviduct ipsilateral to the ovary bearing the dominant follicle[8-10].

There are reports supporting the epidemiologic association of talc use and risk of ovarian cancer[11, 12]. Recent studies have shown that risks for EOC from genital talc use vary by histologic subtype, menopausal status at diagnosis, hormone therapy use, weight, and smoking. These observations suggest that estrogen and/or prolactin may play a role via macrophage activity and inflammatory response to talc. There has been debate as to the significance of the epidemiologic studies based on the fact that the reported epidemiologic risk of talc use and risk of ovarian cancer, although consistent, is relatively modest (30-40%), and there is inconsistent increase in risk with duration of use. This observation is due, in part, to the challenges in quantifying exposure as well as the failure of epidemiological studies to obtain necessary information about the frequency and duration of usage[11-13].

In this study, we have shown beyond doubt that talc alters key redox and inflammatory markers, enhances cell proliferation, and inhibits apoptosis, which are hallmarks of ovarian cancer. More importantly, this effect is also manifested by talc in normal cells, including surface ovarian epithelium, fallopian tube, and macrophages. Oxidative stress has been implicated in the pathogenesis of ovarian cancer, specifically, by increased expression of several key pro-oxidant enzymes such as iNOS, MPO, and NAD(P)H oxidase in EOC tissues and cells as compared to normal cells indicating an enhanced redox state, as we have recently demonstrated[14]. This redox state is further enhanced in chemoresistant EOC cells as evident by a further increase in iNOS and NO_2^-/NO_3^- and a decrease in GSR levels, suggesting a shift towards a pro-oxidant state[14]. Antioxidant enzymes, key regulators of cellular redox balance, are differentially expressed in various cancers, including ovarian[14, 15]. Specifically, GPX expression is reduced in prostate, bladder, kidney, and estrogen receptor negative breast cancer cell lines, though GPX is increased in other cancerous tissues from breast[15]. Glutathione reductase levels, on the other hand, are elevated in lung cancer, although differentially expressed in breast and kidney cancer[5, 16]. Similarly, CAT was decreased in breast, bladder, and lung cancer while increased in brain cancer[17-19]. Superoxide dismutase is expressed in lung, colorectal, gastric ovarian, and breast cancer, while decreased activity and expression have been reported in colorectal carcinomas and pancreatic cancer cells[19-22]. Collectively, this differential expression of antioxidants demonstrates the unique and complex redox microenvironment in

cancer. Glutathione reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH. This enzyme is essential for the GSH redox cycle which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress (Figure 5). Treatment with talc significantly reduced GSR in normal and cancer cells, altering the redox balance (Figure 3. A, C). Likewise, GPX is an enzyme that detoxifies reactive electrophilic intermediates and thus plays an important role in protecting cells from cytotoxic and carcinogenic agents. Overexpression of GPX is triggered by exogenous chemical agents and reactive oxygen species, and is thus thought to represent an adaptive response to stress[16]. Indeed, treatment of normal and cancer cells with talc significantly reduced GPX, which compromised the overall cell response to stress (Figure 3. B, D).

We have previously reported that EOC cells manifest increased cell proliferations and –decreased apoptosis[14]. In this study, we have shown that talc enhances cell proliferation and induces an inhibition in apoptosis in EOC cells, but more importantly in normal cells, suggesting talc is a stimulus to the development of the oncogenic phenotype. We also previously reported a cross-talk between iNOS and MPO in ovarian cancer which contributed to the lower apoptosis observed in ovarian cancer cells[14, 23]. Myeloperoxidase, an abundant hemoprotein, previously known to be present solely in neutrophils and monocytes, is a key oxidant enzyme that utilizes NO produced by iNOS as a one-electron substrate generating NO^+, a labile nitrosylating species[14, 24, 25]. We were the first to report that MPO was expressed by EOC cells and tissues, and that silencing MPO gene expression utilizing MPO specific siRNA induced apoptosis in EOC cells through a mechanism that involved the S-nitrosylation of caspase-3 by MPO[23]. Additionally, we have compelling evidence that MPO serves as a source of free iron under oxidative stress, where both NO^- and superoxide are elevated[14]. Iron reacts with hydrogen peroxide (H_2O_2) and catalyzes the generation of highly reactive hydroxy radical ($HO^\bullet$), thereby increasing oxidative stress, which in turn increases free iron concentrations by the Fenton and Haber–Weiss reaction[14, 25]. We have previously highlighted the potential benefits of the combination of serum MPO and free iron as biomarkers for early detection and prognosis of ovarian cancer[26]. Collectively, we now have substantial

evidence demonstrating that altered oxidative stress may play a role in maintaining the oncogenic phenotype of EOC cells. Treatment of normal or ovarian cancer cells with talc resulted in a significant increase in MPO and iNOS, supporting the role of talc in the enhancement of a pro-oxidant state that is a major cause in the development and maintenance of the oncogenic phenotype (Figure 2).

Furthermore, CA-125, which exists as a membrane-bound and secreted protein in epithelial ovarian cancer cells, has been established as a biomarker for disease progression and response to treatment[2]. CA-125 expression was significantly increased from nearly undetectable levels in controls to values approaching clinical significance (35 U/ml in postmenopausal women[27]) in talc treated cells (Figure 4, $p<0.05$) without the physiologic effects on the tumor microenvironment one would expect to be present in the human body, highlighting the implications of the pro-oxidant states caused by talc alone.

To elucidate the mechanism by which talc alters the redox balance to favor a pro-oxidant state not only in ovarian cancer cells, but more importantly in normal cells, we have examined selected known gene mutations corresponding to SNPs known to be associated with altered enzymatic activity and increased cancer risk[6, 28]. Our results show that the *CAT* SNP (rs769217) which results in decreased enzymatic activity was induced in all normal cell lines tested and in TOV112D EOC lines, but was not detected in A2780 or SKOV-3 cell lines (Table 2). Nevertheless, our results confirm a decrease in CAT expression and enzymatic activity in all talc treated cells (Figure 1), indicating the existence of other *CAT* SNPs. The *SOD3* (rs2536512) and *GSR* (rs8190955) SNP genotypes were not detected in any cell line, yet SOD3 and GSR activity and expression were decreased in all talc treated cells, again suggesting the presence of other SNPs. Our results have also shown that all cells, except for HOSEpiC cells, manifest the SNP genotype of *GPX1* (C/T) before talc treatment. Intriguingly, talc treatment reversed this SNP genotype to the normal genotype (Table 2). Consistent with this finding, we have previously reported that acquisition of chemoresistance by ovarian cancer cells is associated with a switch from the *GPX1* SNP genotype to the normal *GPX1* genotype[6]. It is not understood why a *GPX1* SNP genotype predominates in untreated normal and ovarian cancer cells. Our results showed that talc treatment was associated with a genotype switch from common C/C genotype in *NOS2* in untreated cells to T/T, the SNP genotype, in talc

treated cells, except in A2780 and TOV112D (Table 2). Nevertheless, our results confirm an increase in iNOS expression and enzymatic activity in all talc treated cells (Figure 2), again suggesting the existence of other *NOS2* SNPs. Collectively, these findings support the notion that talc treatment induced gene point mutations that happen to correspond to SNPs in locations with functional effects, thus altering overall redox balance for the initiation and development of ovarian cancer. Future studies examining such SNPs are important to fully elucidate a genotype switch mechanism induced by talc exposure.

In summary, this is the first study to clearly demonstrate that talc induces inflammation and alters the redox balance favoring a pro-oxidant state in normal and EOC cells. We have shown a dose-dependent significant increase in key pro-oxidants, iNOS, $NO2^-/NO3^-$, and MPO and a concomitant decrease in key antioxidant enzymes, CAT, SOD, GPX, and GSR, in all talc treated cells (both normal and ovarian cancer) compared to their controls. Additionally, there was a significant increase in CA-125 levels in all the talc treated cells compared to their controls, except in macrophages. The mechanism by which talc alters the cellular redox and inflammatory balance involves the induction of specific mutations in key oxidant and antioxidant enzymes that correlate with alterations in their activities. The fact that these mutations happen to correspond to known SNPs of these enzymes indicate a genetic predisposition to developing ovarian cancer with genital talcum powder use.

Acknowledgements

Special thanks to Imaan Singh for her technical contributions in acquiring the data and in development of graphics.

Conflict of Interest

The corresponding author, Dr. Ghassan Saed, acted as a consultant regarding this topic for a fee, otherwise, the authors declare that there are no conflicts of interest.

References

1. Berek JS, Bertelsen K, du Bois A, et al. [Epithelial ovarian cancer (advanced stage): consensus conference (1998)]. Gynecologie, obstetrique & fertilite. 2000 Jul-Aug;**28**(7-8):576-83.

2. Jelovac D, Armstrong DK. Recent progress in the diagnosis and treatment of ovarian cancer. CA: a cancer journal for clinicians. 2011 May-Jun;**61**(3):183-203.

3. Prat J, Ribe A, Gallardo A. Hereditary ovarian cancer. Hum Pathol. 2005 Aug;**36**(8):861-70.

4. Ramus SJ, Vierkant RA, Johnatty SE, et al. Consortium analysis of 7 candidate SNPs for ovarian cancer. Int J Cancer. 2008 Jul 15;**123**(2):380-8.

5. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med. 2010 Dec 1;**49**(11):1603-16.

6. Fletcher NM, Belotte J, Saed MG, et al. Specific point mutations in key redox enzymes are associated with chemoresistance in epithelial ovarian cancer. Free Radic Biol Med. 2016 Nov 25;**102**:122-32.

7. Saed GM, Diamond MP, Fletcher NM. Updates of the role of oxidative stress in the pathogenesis of ovarian cancer. Gynecologic oncology. 2017 Jun;**145**(3):595-602.

8. Kunz G, Beil D, Deiniger H, Einspanier A, Mall G, Leyendecker G. The uterine peristaltic pump. Normal and impeded sperm transport within the female genital tract. Adv Exp Med Biol. 1997;**424**:267-77.

9. Leyendecker G, Kunz G, Herbertz M, et al. Uterine peristaltic activity and the development of endometriosis. Ann N Y Acad Sci. 2004 Dec;**1034**:338-55.

10. Zervomanolakis I, Ott HW, Hadziomerovic D, et al. Physiology of upward transport in the human female genital tract. Ann N Y Acad Sci. 2007 Apr;**1101**:1-20.

11. Terry KL, Karageorgi S, Shvetsov YB, et al. Genital powder use and risk of ovarian cancer: a pooled analysis of 8,525 cases and 9,859 controls. Cancer Prev Res (Phila). 2013 Aug;**6**(8):811-21.

12. Penninkilampi R, Eslick GD. Perineal Talc Use and Ovarian Cancer: A Systematic Review and Meta-Analysis. Epidemiology. 2018 Jan;**29**(1):41-9.

13. Reid BM, Permuth JB, Sellers TA. Epidemiology of ovarian cancer: a review. Cancer Biol Med. 2017 Feb;**14**(1):9-32.

14. Saed GM, Diamond MP, Fletcher NM. Updates of the role of oxidative stress in the pathogenesis of ovarian cancer. Gynecologic oncology. 2017 Feb 23.

15. Brigelius-Flohe R, Kipp A. Glutathione peroxidases in different stages of carcinogenesis. Biochimica et biophysica acta. 2009 Nov;**1790**(11):1555-68.

16. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. Free Radic Biol Med. 1990;**8**(6):583-99.

17. Popov B, Gadjeva V, Valkanov P, Popova S, Tolekova A. Lipid peroxidation, superoxide dismutase and catalase activities in brain tumor tissues. Arch Physiol Biochem. 2003 Dec;**111**(5):455-9.

18. Ray G, Batra S, Shukla NK, et al. Lipid peroxidation, free radical production and antioxidant status in breast cancer. Breast Cancer Res Treat. 2000 Jan;**59**(2):163-70.

19. Chung-man Ho J, Zheng S, Comhair SA, Farver C, Erzurum SC. Differential expression of manganese superoxide dismutase and catalase in lung cancer. Cancer Res. 2001 Dec 1;**61**(23):8578-85.

20. Radenkovic S, Milosevic Z, Konjevic G, et al. Lactate dehydrogenase, catalase, and superoxide dismutase in tumor tissue of breast cancer patients in respect to mammographic findings. Cell Biochem Biophys. 2013 Jun;**66**(2):287-95.

21. Hu Y, Rosen DG, Zhou Y, et al. Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: role in cell proliferation and response to oxidative stress. J Biol Chem. 2005 Nov 25;**280**(47):39485-92.

22. Svensk AM, Soini Y, Paakko P, Hiravikoski P, Kinnula VL. Differential expression of superoxide dismutases in lung cancer. Am J Clin Pathol. 2004 Sep;**122**(3):395-404.

23. Saed GM, Ali-Fehmi R, Jiang ZL, et al. Myeloperoxidase serves as a redox switch that regulates apoptosis in epithelial ovarian cancer. Gynecologic oncology. 2010 Feb;**116**(2):276-81.

24. Galijasevic S, Saed GM, Hazen SL, Abu-Soud HM. Myeloperoxidase metabolizes thiocyanate in a reaction driven by nitric oxide. Biochemistry. 2006 Jan 31;**45**(4):1255-62.

25. Galijasevic S, Maitra D, Lu T, Sliskovic I, Abdulhamid I, Abu-Soud HM. Myeloperoxidase interaction with peroxynitrite: chloride deficiency and heme depletion. Free Radic Biol Med. 2009 Aug 15;**47**(4):431-9.

26. Fletcher NM, Jiang Z, Ali-Fehmi R, et al. Myeloperoxidase and free iron levels: potential biomarkers for early detection and prognosis of ovarian cancer. Cancer Biomark. 2011;**10**(6):267-75.

27. Scholler N, Urban N. CA125 in ovarian cancer. Biomark Med. 2007 Dec;**1**(4):513-23.

28. Belotte J, Fletcher NM, Saed MG, et al. A Single Nucleotide Polymorphism in Catalase Is Strongly Associated with Ovarian Cancer Survival. PloS one. 2015;**10**(8):e0135739.

Table Legends:

Table 1. Real-time RT-PCR oligionucleotide primers.

Table 2. SNP characteristics (A) and SNP genotyping of key redox enzymes in untreated and talc treated (100 μg/mL) human primary ovarian epithelial cells (Normal ovarian), Human Ovarian Surface Epithelial Cells (HOSEpiC) , fallopian tube (FT33), and ovarian cancer (A2780, SKOV-3, TOV112D) cell lines (B).

Figure Legends:

Figure 1. Decreased expression and activity of key antioxidant enzymes, CAT and SOD3. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of CAT (A&C) and SOD3 (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells and in all doses as compared to controls.

Figure 2. Increased expression and activity of key pro-oxidants, iNOS, NO_2^-/NO_3^-, and MPO. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of iNOS (A&C) and MPO (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. As expected, there was no detectable MPO in normal ovarian and fallopian tube cells, and thus talc treatment did not have any effect. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in iNOS and MPO positive cells and in all doses as compared to controls.

Figure 3. Decreased expression and activity of key antioxidant enzymes, GSR and GPX. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of GSR (A&C) and GPX (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells and in all doses as compared to controls.

Figure 4. Increased CA125 levels in response to talc treatment. The level of ovarian cancer biomarker CA-125 was determined by ELISA before and after 72 hours of talc treatment (100 μg/ml) in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cells. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells as compared to controls.

Figure 5. Increased cell proliferation in response to talc treatment. Cell proliferation was determined by MTT Cell Proliferation Assay after 72 hours of talc treatment (100 µg/ml) in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cells. Experiments were performed in triplicate. Cell proliferation is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells as compared to controls.

Figure 6. Decreased apoptosis in response to talc treatment. Caspase-3 activity was used to measure the degree of apoptosis in all cells. Caspase-3 Activity Assay was utilized to determine caspase-3 activity in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard error. All changes in response to talc treatment were significant ($p<0.05$) in all cells and in all doses as compared to controls.

Figure 7. Epithelial ovarian cancer (EOC) cells have been reported to manifest a persistent pro-oxidant state as evident by the upregulation (green arrows) of key oxidants iNOS, NO, NO+, ONOO-, OH-, O2+, and MPO (blue) and downregulation (red arrows) of key antioxidants SOD, CAT, GPX, and GSR (orange). This redox state was also shown to be further enhanced in chemoresistant EOC cells. In this study, talcum powder altered the redox state, as indicated by the arrows, of both normal and EOC cells to create an enhanced pro-oxidant state.

Accession Number	Gene	Sense (5'-3')	Antisense (3'-5')	Amplicon (bp)	Annealing Time (sec) and Temperature (°C)
NM_001101	*β-actin*	ATGACTTAGTTGCGTTACAC	AATAAAGCCATGCCAATCTC	79	10, 64
NM_001752	*CAT*	GGTTGAACAGATAGCCTTC	CGGTGAGTGTCAGGATAG	105	10, 63
NM_003102	*SOD3*	GTGTTCCTGCCTGCTCCT	TCCGCCGAGTCAGAGTTG	84	60, 64
NM_000637	*GSR*	TCACCAAGTCCCATATAGAAATC	TGTGGCGATCAGGATGTG	116	10, 63
NM_000581	*GPX1*	GGACTACACCCAGATGAAC	GAGCCCTTGCGAGGTGTAG	91	10, 66
NM_000625	*NOS2*	GAGGACCACATCTACCAAGGAGGAG	CCAGGCAGGCGGAATAGG	89	30, 59
NM_000250	*MPO*	CACTTGTATCCTCTGGTTCTTCAT	TCTATATGCTTCTCACGCCTAGTA	79	60, 63

A	Gene (rs number)				
	CAT (rs769217)	*NOS2* (rs2297518)	*GSR* (rs8190955)	*GPX1* (rs3448)	*SOD3* (rs2536512)
MAF	0.123	0.173	0.191	0.176	0.476
SNP	C-262T	C2087T	G201T	C-1040T	A377T
Chromosome Location	11p13	17q11.2	8p12	3q21.31	4p15.2
Amino Acid Switch	Isoleucine to Threonine	Serine to Leucine	Unknown	Unknown	Alanine to threonine
Effect on Activity	Decrease	Increase	Unknown	Unknown	Decrease

B	Gene (rs number)				
Cell Lines	*CAT* (rs769217)	*NOS2* (rs2297518)	*GSR* (rs8190955)	*GPX1* (rs3448)	*SOD3* (rs2536512)
A2780- Control	C/C	C/C	G/G	**C/T**	A/A
A2780- Talc	C/C	C/C	G/G	C/C	A/A
SKOV-3- Control	C/C	C/C	G/G	**C/T**	A/A
SKOV-3- Talc	C/C	**T/T**	G/G	C/C	A/A
TOV112D- Control	C/C	C/C	G/G	**C/T**	A/A
TOV112D-Talc	**C/T**	C/C	G/G	C/C	A/A
HOSEpiC- Control	C/C	C/C	G/G	**C/T**	A/A
HOSEpiC- Talc	**C/T**	**T/T**	G/G	**C/T**	A/A
FT33- Control	C/C	C/C	G/G	**C/T**	A/A
FT33- Talc	**C/T**	**T/T**	G/G	C/C	A/A
Normal Ovarian- Control	C/C	C/C	G/G	**C/T**	A/A
Normal Ovarian- Talc	**C/T**	**T/T**	G/G	C/C	A/A

Table 2. SNP characteristics (A) and SNP genotyping of key redox enzymes in untreated and talc treated (100 µg/mL) human primary ovarian epithelial cells (Normal ovarian), Human Ovarian Surface Epithelial Cells (HOSEpiC) , fallopian tube (FT33), and ovarian cancer (A2780, SKOV-3, TOV112D) cell lines (B).

254x190mm (300 x 300 DPI)

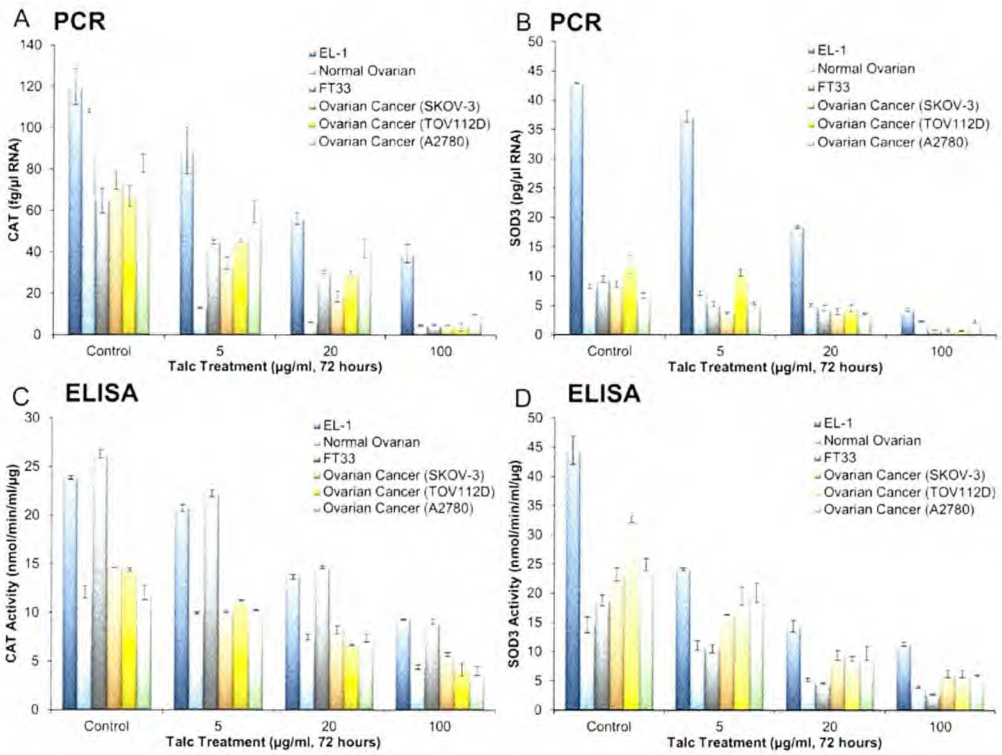

Figure 1. Decreased expression and activity of key antioxidant enzymes, CAT and SOD3. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of CAT (A&C) and SOD3 (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells and in all doses as compared to controls.

254x190mm (300 x 300 DPI)

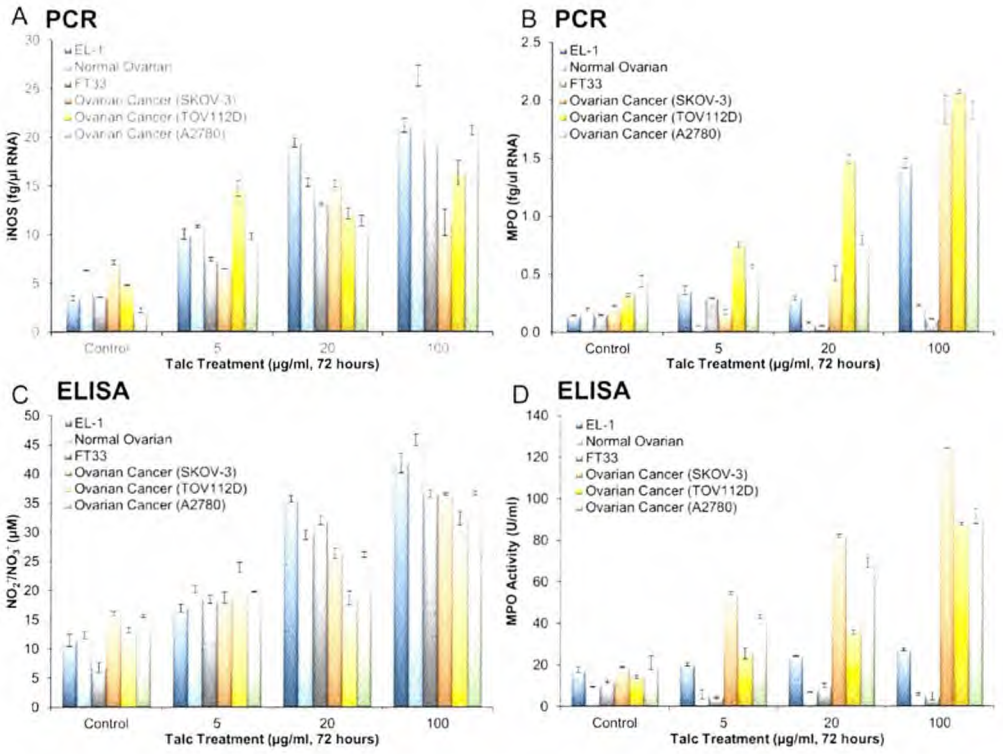

Figure 2. Increased expression and activity of key pro-oxidants, iNOS, NO2-/NO3-, and MPO. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of iNOS (A&C) and MPO (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. As expected, there was no detectable MPO in normal ovarian and fallopian tube cells, and thus talc treatment did not have any effect. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in iNOS and MPO positive cells and in all doses as compared to controls.

254x190mm (300 x 300 DPI)

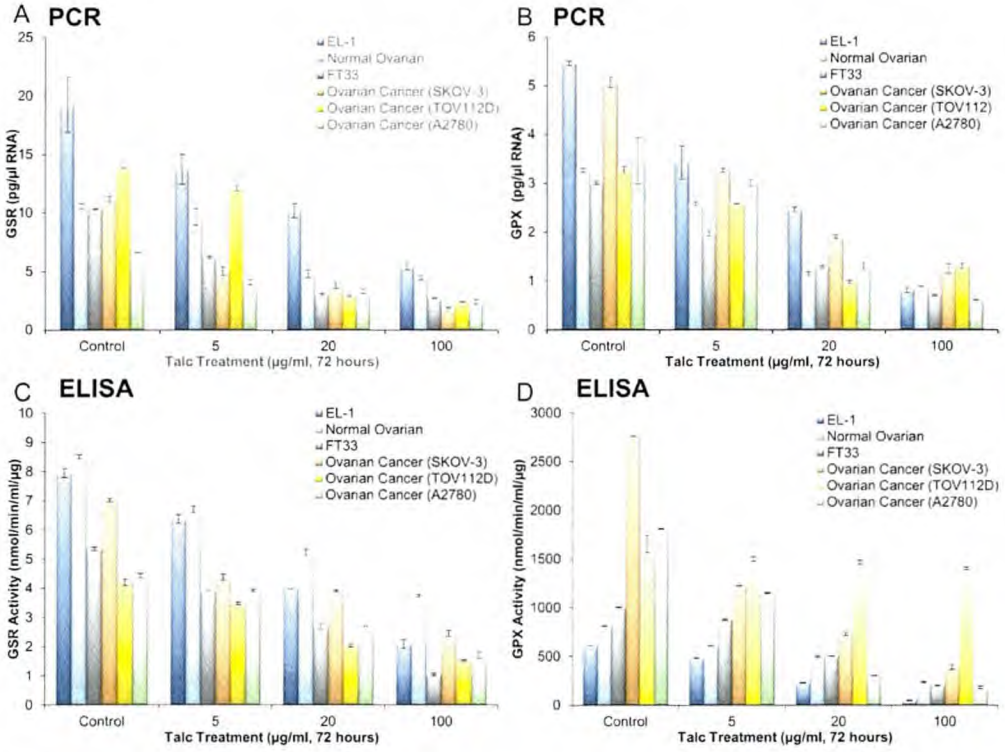

Figure 3. Decreased expression and activity of key antioxidant enzymes, GSR and GPX. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of GSR (A&C) and GPX (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells and in all doses as compared to controls.

254x190mm (300 x 300 DPI)

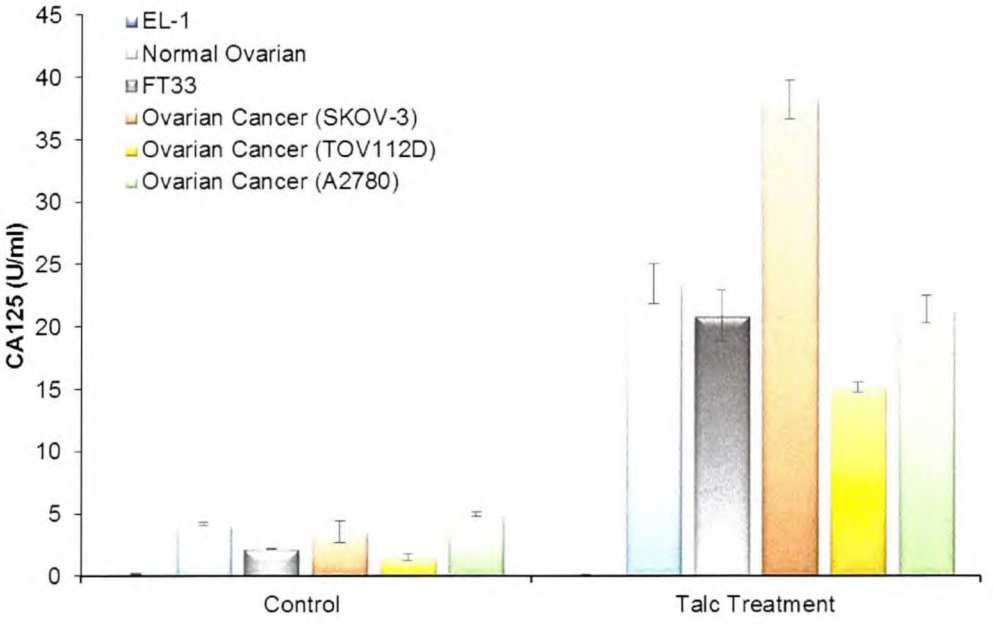

Figure 4. Increased CA125 levels in response to talc treatment. The level of ovarian cancer biomarker CA-125 was determined by ELISA before and after 72 hours of talc treatment (100 µg/ml) in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cells. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells as compared to controls.

74x47mm (300 x 300 DPI)

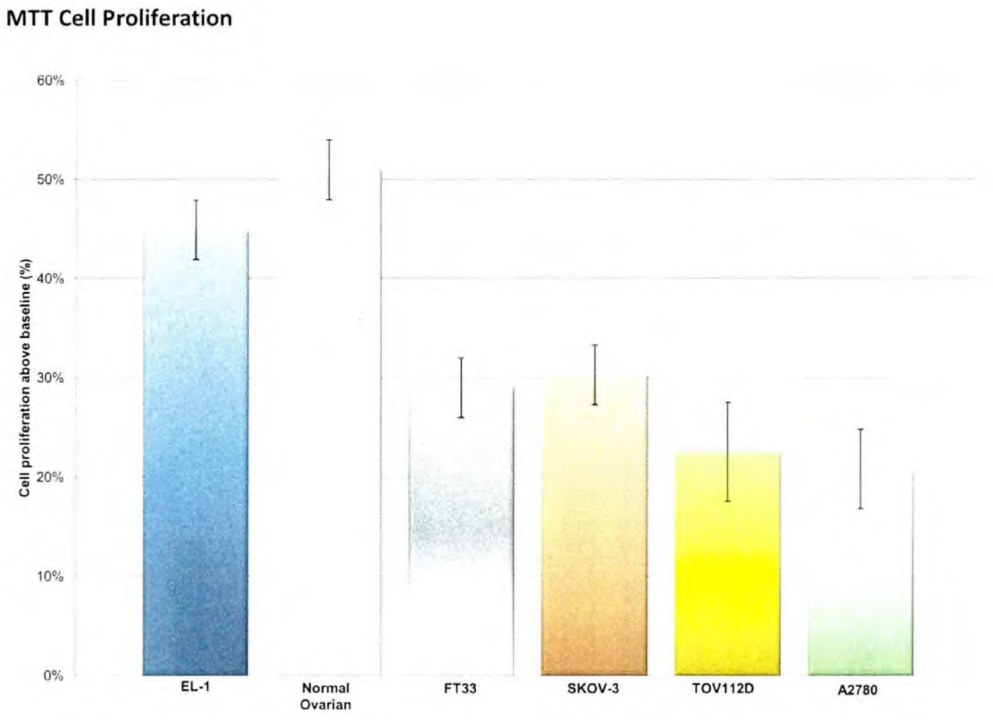

Figure 5. Increased cell proliferation in response to talc treatment. Cell proliferation was determined by MTT Cell Proliferation Assay after 72 hours of talc treatment (100 µg/ml) in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cells. Experiments were performed in triplicate. Cell proliferation is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells as compared to controls.

254x190mm (300 x 300 DPI)

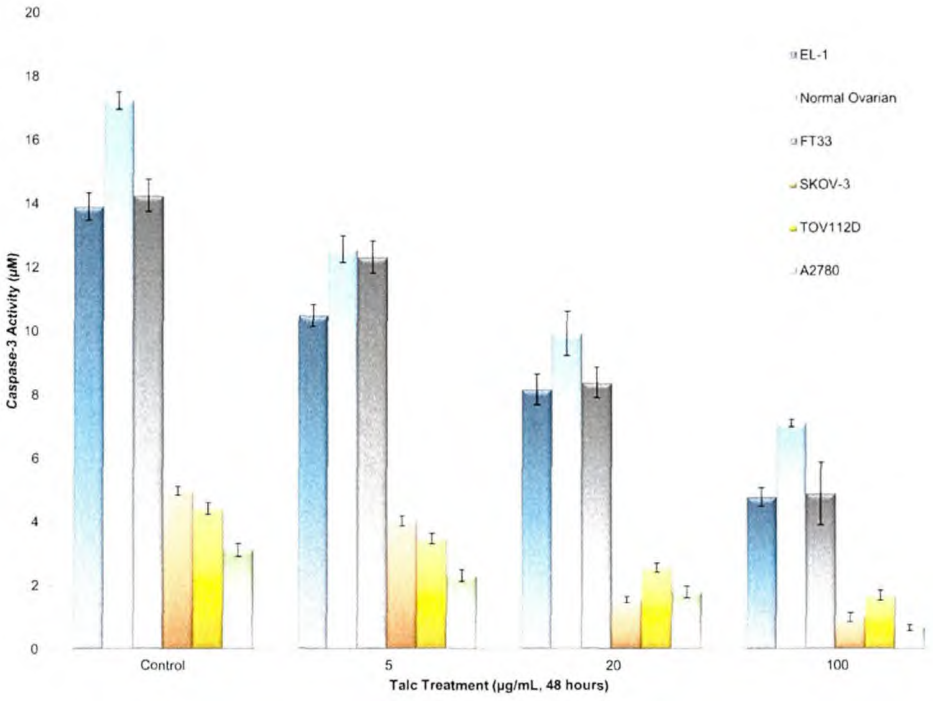

Figure 6. Decreased apoptosis in response to talc treatment. Caspase-3 activity was used to measure the degree of apoptosis in all cells. Caspase-3 Activity Assay was utilized to determine caspase-3 activity in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard error. All changes in response to talc treatment were significant ($p<0.05$) in all cells and in all doses as compared to controls.

254x190mm (300 x 300 DPI)

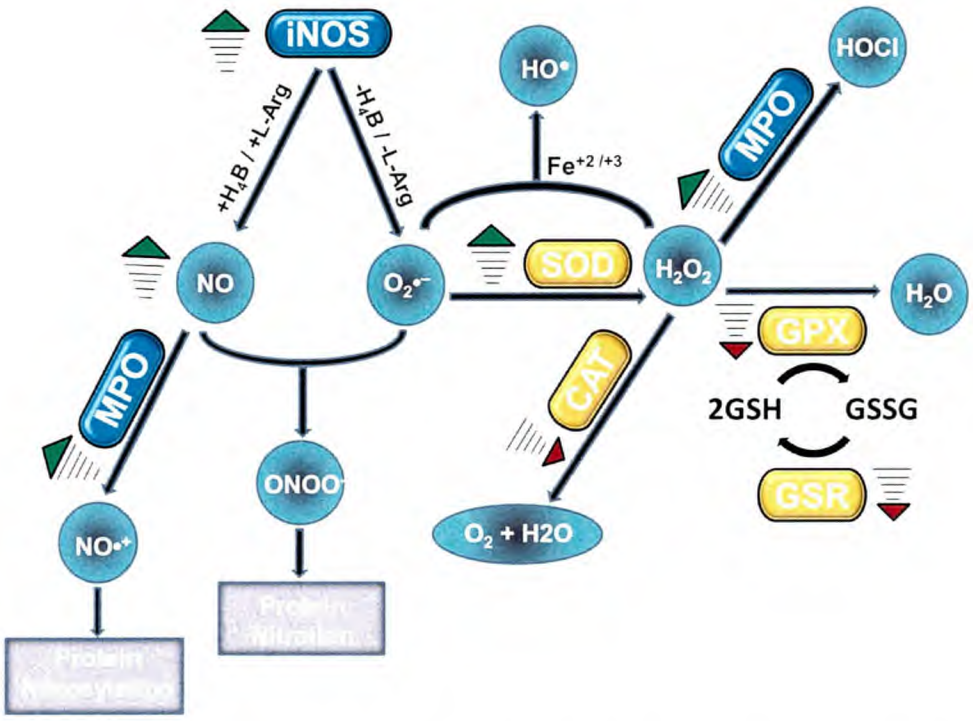

Figure 7. Epithelial ovarian cancer (EOC) cells have been reported to manifest a persistent pro-oxidant state as evident by the upregulation (green arrows) of key oxidants iNOS, NO, NO+, ONOO-, OH-, O2+, and MPO (blue) and downregulation (red arrows) of key antioxidants SOD, CAT, GPX, and GSR (orange). This redox state was also shown to be further enhanced in chemoresistant EOC cells. In this study, talcum powder altered the redox state, as indicated by the arrows, of both normal and EOC cells to create an enhanced pro-oxidant state.

254x190mm (300 x 300 DPI)